JOURNAL OF FUNCTIONAL FOODS 13 (2015) 225–238

Available online at www.sciencedirect.com

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Characterization and comparative assessment of antioxidant and ACE inhibitory activities of thornback ray gelatin hydrolysates

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ARTICLE INFO

Article history:
Received 17 September 2014
Received in revised form 22 December 2014
Accepted 23 December 2014
Available online 21 January 2015

Keywords:
Thornback ray (*Raja clavata*)
Gelatin hydrolysate
ACE inhibitory activity
Antioxidative activity

ABSTRACT

The angiotensin I-converting enzyme (ACE) inhibitory activities and antioxidant properties of thornback ray gelatin hydrolysates (TRGHs) prepared by treatment with proteolytic proteases from *Bacillus subtilis* A26, *Raja clavata* crude alkaline protease extract, Alcalase and Neutrase were investigated. All gelatin hydrolysates showed different degrees of hydrolysis and hydrophobic/hydrophilic peptides ratio. Moreover, they possess high protein content (70.04 ± 0.55–74.14 ± 0.28%). The antioxidant activity was assayed using various *in vitro* tests. The highest antioxidant activity was observed with hydrolysate obtained by treatment with A26 proteases (TRGH-A26) which exhibited a 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity with a concentration that produces 50% of inhibition (IC_{50}) of 1.98 ± 0.02 mg/ml of sample, reduced the ferric ions with an absorbance at 700 nm of 0.962 ± 0.07, prevents bleaching of β-carotene with 73.02 ± 1.90% inhibition and gave an antioxidative efficacy of 180 ± 0.08 μmol/ml α-tocopherol equivalents at 5 mg/ml in the phosphomolybdenum assay. However, gelatin hydrolysate treated with Alcalase (TRGH-Alcalase) was the most potent to prevent DNA oxidation. For the ACE inhibitory activity, all hydrolysates displayed ACE-inhibitory activity. TRGH-A26 and TRGH-Alcalase exhibited the highest activity with 85 ± 0.65 and 82 ± 0.49%, respectively, at 5 mg/ml. The results revealed that TRGHs could be used as ingredients to formulate functional foods.

1. Introduction

Reactive oxygen species (ROS), such as the superoxide radical anion, hydroxyl radical and nitric oxide, generated during oxidative stress conditions cause adverse effect to cells if they are overproduced (Buonocore, Perrone, & Tataranno, 2010). Therefore, the production of ROS should be firmly regulated. This regulation is achieved by the antioxidant systems in the human body such as antioxidant enzymes and

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http://dx.doi.org/10.1016/j.jff.2014.12.042
1756-4646/

nonenzymatic factors, which maintain the balance between oxidants and antioxidants. However, this balance could be disturbed under pathological conditions, so supplementary antioxidants are needed to mediate the oxidative balance, in order to protect the cells from oxidation (Kim, Je, & Kim, 2007). Many diseases are caused by excessive production of ROS such as brain dysfunction, cancer, decline of the immune system and heart diseases (Aruoma, 1998). Additionally, lipid peroxidation occurring in food products is of great concern to the food industry and consumers, because it can lead to the deterioration of food quality by development of undesirable off-flavours, decreasing the shelf life and formation of potentially toxic reaction products (Pihlanto, 2006).

Synthetic antioxidants such as t-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate have generally been used in food products to delay the deterioration caused by lipid oxidation (Winata & Lorenz, 1996). However, these antioxidants have been found to exhibit various undesirable health effects. Therefore, there is great interest in finding new and safe antioxidants from natural sources. Recently, antioxidant activity has been reported in many different sources of proteins hydrolysates, such as goby protein (Nasri et al., 2014), porcine plasma protein (Liu, Kong, Xiong, & Xia, 2010) and eggs' protein (Sakanaka, Tachibana, Ishihara, & Juneja, 2004).

Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of human blood pressure and fluid homeostasis via the renin angiotensin system (RAS). ACE hydrolyses angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into a powerful vasoconstrictor, angiotensin II, by removing the C-terminal dipeptide His-Leu (Lavoie & Sigmund, 2003). In addition, ACE inactivates the bradykinin which is a vasodilator. Potent ACE inhibitory hydrolysates and peptides have been isolated from different food sources such as sea cucumber gelatin (Zhao et al., 2007), rice (Chen et al., 2013) and skipjack roe (Intarasirisawat, Benjakul, Wu, & Visessanguan, 2013). Such bioactive peptides are inactive in their original protein, and can be released to active form through the intestinal digestion or hydrolysis by proteases (Wu et al., 2008). Furthermore, many studies on antioxidative and ACE inhibitory activities have been performed from fish skin gelatin hydrolysates such as squid (Alemán et al., 2011), pacific cod (Himaya, Ngo, Ryu, & Kim, 2012) and blacktip shark gelatin (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2013).

Thornback ray, is a widely distributed skate (Rajiformes: Rajidae) in the eastern Atlantic, ranging from Norway and Iceland to Northwest Africa, including the Mediterranean and Black Seas. They may also occur in the Atlantic and Indian Oceans of southern Africa (Stehmann & Bürkel, 1994). During processing *Raja clavata* generates a significant amount of by-products including skin, tail, head and viscera (80 g/100 g fresh weight) (Port Authority of Vigo, 2008). In the present study, different gelatin hydrolysates were elaborated from the skin of thornback ray (*R. clavata*) by the use of different types of enzyme. Hydrophobicity, physico-chemical and amino acid composition of the hydrolysates were investigated as well as their antioxidative and ACE-inhibitory activities. Proteomic identification of peptides by using matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) was also studied.

2. Materials and methods

2.1. Reagents

Butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene, linoleic acid and angiotensin-converting enzyme (ACE from rabbit lung), α-tocopherol and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Abz-Gly-*p*-nitro-phe-pro-OH trifluoroacetate salt was obtained from Bachem AG. (Bubendorf, Switzerland). Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade. All other chemicals, namely potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, l-ascorbic acid and sodium hydroxide were of analytical grade.

2.2. Materials

Fresh thornback rays (*R. clavata*) with length of 98–120 cm were obtained in February, from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice and transported to the laboratory within 30 min. Intestines were separated from the other parts of the viscera and then stored in sealed plastic bags at –80 °C until they were used for enzyme extraction, less than one week later. Skins were collected and residual meat was removed manually. Cleaned skins were washed with tap water, cut with scissors into small pieces (0.5 × 0.5 cm) and placed in polyethylene bags. Skins were used immediately or stored at –80 °C until use less than three months.

2.3. Proteolytic enzymes

Alcalase® 2.4L serine-protease from *Bacillus licheniformis* and Neutrase® 0.5L metallo-protease from *Bacillus amyloliquefaciens* were supplied by Novozymes® (Bagsvaerd, Denmark). Crude enzyme preparation from *Bacillus subtilis* A26 was prepared in our laboratory (Agrebi et al., 2009). Crude alkaline protease extract from *R. clavata* was prepared according to the following protocol: intestines (100 g) were homogenized for 3 min with 100 ml of extraction buffer (10 mM Tris–HCl, pH 8.0) (w/v = 1) with the use of Moulinex® R62 homogenizer. The resulting preparation was centrifuged at 8500 *g* for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and used as crude alkaline protease extract. The method of Kembhavi, Kulkarni, and Pant (1993) was used to measure the alkaline protease activity.

2.4. Preparation of thornback ray gelatin hydrolysates (TRGHs)

Thornback ray gelatin (TRG) was extracted from the skin wastes following the method described by Lassoued et al. (2014). Glycine–HCl buffer (100 mmol/l, pH 2.0) in combination with commercial pepsin (MP Biomedicals, Strasbourg, France) were used in the extraction process.

To elaborate the different thornback ray gelatin hydrolysates (TRGHs), the gelatin powder was dissolved in distilled water at 2.5% (w/v), adjusted to the appropriate pH and temperature of each enzyme: Alcalase (8.0, 50 °C), Neutrase (7.0, 50 °C), enzyme